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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Increasing evidence from our lab and others supports that ribosomal proteins play a critical role in development(1) and diseases including bone marrow disease(2) in addition to its essential role in protein synthesis. We found Rpl22 is dispensable for protein biosynthesis but regulates transformation and hematopoiesis(1, 3). Previously I have determined that Rpl22 functions as a haploinsufficient tumor suppressor in a mouse T-cell lymphoma model by activating NFκB and its target Lin28B(3). Recently we also found that Rpl22 knockout mice exhibit an MDS-like phenotype associated with anemia and abnormal bone marrow (BM) hematopoiesis. Consistent with what we observed in our mouse model, our collaborator found that Rpl22 was mutated or deleted in some MDS and AML patients. By using shRNA targeting ER stress proteins and other ribosomal proteins, we found that loss of Rpl22, but not other ribosomal proteins, induces Lin28B and the activation of NFκB and Lin28B mainly depends on ER stress signaling through PERK. On the other hand, Rpl22 has a homolog Rpl22-Like1 (Like1) that is induced upon Rpl22 inactivation. Therefore, I also investigated whether Like1 is critical in transformation and Lin28B induction and found that indeed Like1 is required for both transformation and NFκB-mediated Lin28B induction. These data suggest that Like1 induction occurs before Lin28B induction and we are now investigating role of Like1 in transformation and perturbation of hematopoiesis. To evaluate the role of Rpl22 and its targets in progression of AML, we introduced AML oncogenic fusion MLL-AF9 into BM transplant mouse model, and found that Rpl22 inactivation accelerates AML progression and correlated with poor survival as we expected. We are still in the progress of investigate Rpl22 in MDS/AML and hopefully can find out new therapeutic target through these efforts.

15. SUBJECT TERMS
Rpl22, MDS, AML, MLL-AF9, ER stress, Rpl22-Like1

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Table of Contents

INTRODUCTION

Ribosomal proteins (RPs) have been well known to be essential for protein synthesis. Recently increasing evidence from our lab and others supports that RP play a critical but poorly understood role in development as well as disease. Mutations in RP cause a group of diseases collectively termed ribosomopathies, which are mainly bone marrow diseases such as MDS and are often associated with increased risk for cancer development. We are interested in Rpl22, an RNA-binding component of the 60S ribosomal subunit. Rpl22 is dispensable for protein biosynthesis but plays a role in regulating transformation and hematopoiesis(1, 3). Previously I have determined that Rpl22 functions as a haploinsufficient tumor suppressor, whose mono-allelic inactivation can accelerate the development of T-cell lymphoma in a mouse model where disease is driven by an MyrAkt2-transgene. Rpl22 inactivation predisposes to transformation by activating the NFκB and its direct target, the stem cell factor Lin28B(3). In addition, we also found that Rpl22 knockout mice exhibit an MDS-like phenotype associated with anemia and abnormal bone marrow (BM) hematopoiesis. Consistent with what we observed in our mouse model, our collaborator found that Rpl22 was mutated or deleted in some MDS and AML patients. Based on these data, I intend to investigate the role of Rpl22 in MDS and its predisposition of AML and hopefully can find out new therapeutic target through these efforts. Rpl22 has a paralog Rpl22-like1 (Like1) that shares 70% sequence identity with Rpl22(1, 4), and I will also explore the role of Like1 in the predisposition of blood malignancies as well.

KEYWORDS

Rpl22, MDS, AML, MLL-AF9, ER stress, Rpl22-Like1

OVERALL PROJECT SUMMARY

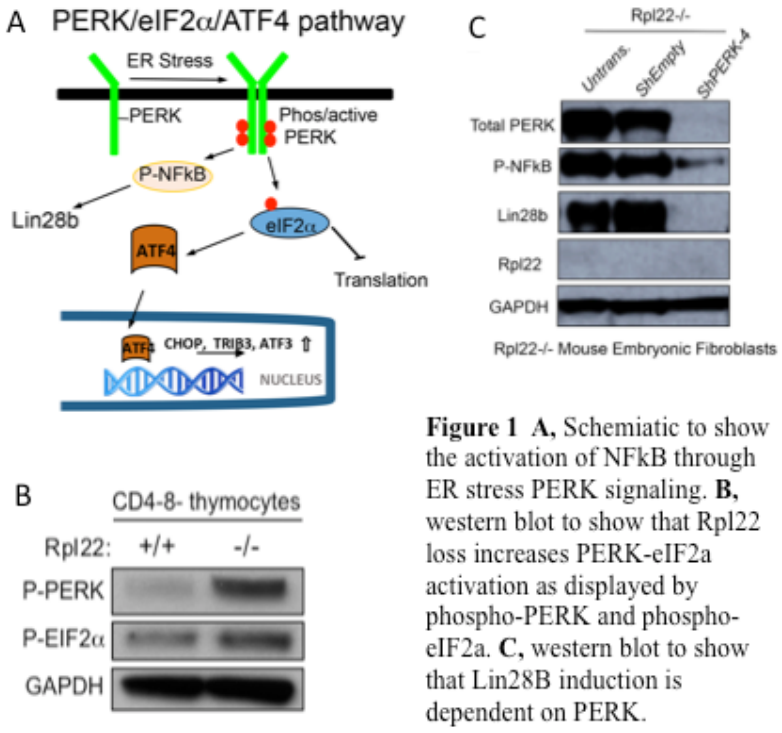

Figure 1 A, Schemiatic to show the activation of NFkB through ER stress PERK signaling. **B,** western blot to show that Rpl22 loss increases PERK-eIF2a activation as displayed by phospho-PERK and phospho-eIF2a. **C,** western blot to show that Lin28B induction is dependent on PERK.

1. *To explore the molecular basis for Lin28B induction by inactivation of Rpl22*

1A: Test whether Rpl22 loss activates NFκB through endoplasmic reticulum stress pathway PERK-phospho-eIF2α-IκBα or IRE1α-TRAF-IKKβ

Recent publications have showed ER stress activation of NFκB signaling(5) and we observed increased ER stress response in Rpl22-deficient cells as demonstrated here (Fig.1B) by increased phospho-PERK and its target phospho-EIF2α. Therefore, we utilized retroviral-mediated shRNA targeting different ER stress components including PERK, IRE1α, and ATF6

to determine which signaling pathway is required for Lin28B induction. As shown in Fig.1C, knockdown of PERK significantly reduced NFκB activation as displayed by phospho-*NFκB* component and Lin28B induction. We did not see significant differences upon ATF6 knockdown. These data support that PERK signaling is responsible the activation NFκB activation and Lin28B induction.

1B. *NFκB/Lin28B induction caused by Rpl22 inactivation also relies on Rpl22 paralog Rpl22-Like1*

I also made an interesting finding that links NFκB/Lin28B induction upon Rpl22 loss with Rpl22 papralog, Rpl22-Like1, (Like1) expression. I have found that Like1 is induced upon Rpl22 inactivation (Fig.2A). Knockdown Like1 inhibits Lin28B induction (Fig.2B) whereas overexpression of Like1 increases Lin28B levels as well as NFκB signaling as demonstrated by phosphorylation of p65 (Fig.2C). We don't know whether PERK activation depends on Like1 induction yet. We are now investigating whether Like1 is required for ER stress to activate NFκB signaling and Lin28B induction. Furthermore, I also observed Like1 induction is required and sufficient to promote transformation in colony formation assay (Fig.2D-E; and not shown). All these data suggest that Like1 induction occurs before Lin28B induction and plays critical in transformation. We are now focusing on the role of Like1 in the progression of blood malignancies(6, 7) and other solid tumors(8) because Like1 amplification was found in these diseases.

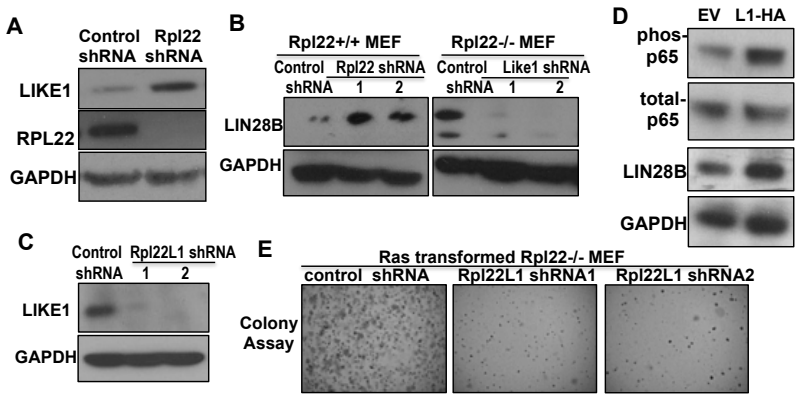

Figure 2. Lin28B induction and transformation induced by Rpl22 inactivaiton depends on Rpl22 paralog Like1. A, western blot to show Like1 induction upon Rpl22 knockdown by shRNA. **B,** western blot to show that Rpl22 loss leads to Lin28B induction and knockdown of Like1 abrogates Lin28B induction. **C**, western to shown knockdown of Like1 by two different shRNAs. **D,** western blot to show that Like1 overexpression (L1-HA) increases NFκB signaling and Lin28B induction. GAPDH is used as loading control for all western. **E**, colony formation assay to show that knockdown of Like1 abrogates transformation caused by Rpl22 loss.

1C. To determine whether inactivation of other ribosomal proteins increases Lin28B

By knockdown other ribosomal proteins with lentiviral-mediated shRNA targeting Rps7, Rpl36, Rpl36a, Rps19 and Rpl14, we failed to observe significant induction of Lin28B as determined by realtime PCR (Fig.2). These data suggest that regulation of Lin28B is specific with Rpl22.

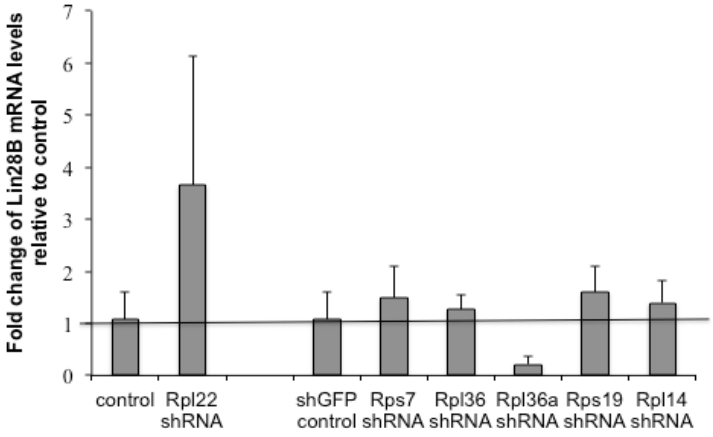

Figure 3. Lin28B mRNA levels were determined by realtime PCR after 72 hours of shRNA infection targeting risbosomal protein Rps7, Rpl36, Rpl36a, Rps19 and Rpl14.

2. *To assess the role of p53 induction in the perturbation of hematopoiesis observed in Rpl22-/- mice*

Ribosomal protein defects frequently induce p53 (9, 10). To assess the role of p53 in perturbation of hematopoiesis in Rpl22-/- mice, p53 null mice were crossed with Rpl22-/- mice. We looked p53 levels in the bone marrow from Rpl22 wild type (WT) and knockout (KO) mice and found out p53 is not dramatically altered in bone marrow cells, particularly upon enriching for stem/progenitor cells by depleting cells expressing lineage markers (Fig.4). We also analyzed the phenotype in Rpl22-/-p53-/- mice and found the mice exhibit similar phenotype with Rpl22-/-. We also did RNA-seq analysis and real-time PCR in the bone marrow cells from Rpl22 WT and Rpl22 KO and found out the p53 targets not altered significantly except p21. Based on this, we assume that p53 may not significantly influence hematopoiesis in Rpl22 KO mice.

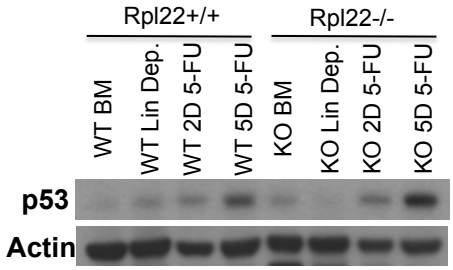

Figure 4. Western blot to show p53 expression levels in bone marrow cells (BM) isolated from Rpl22 wild type (WT) or knockout mice (KO) with or without lineage depletion (Lin Dep.) and 2 days or 5 days 5-Fu treatment. Actin is used as loading control.

3. *To explore whether Rpl22 loss induction accelerate Myelodysplastic Syndrome /Acute Myelogenous Leukemia progression*

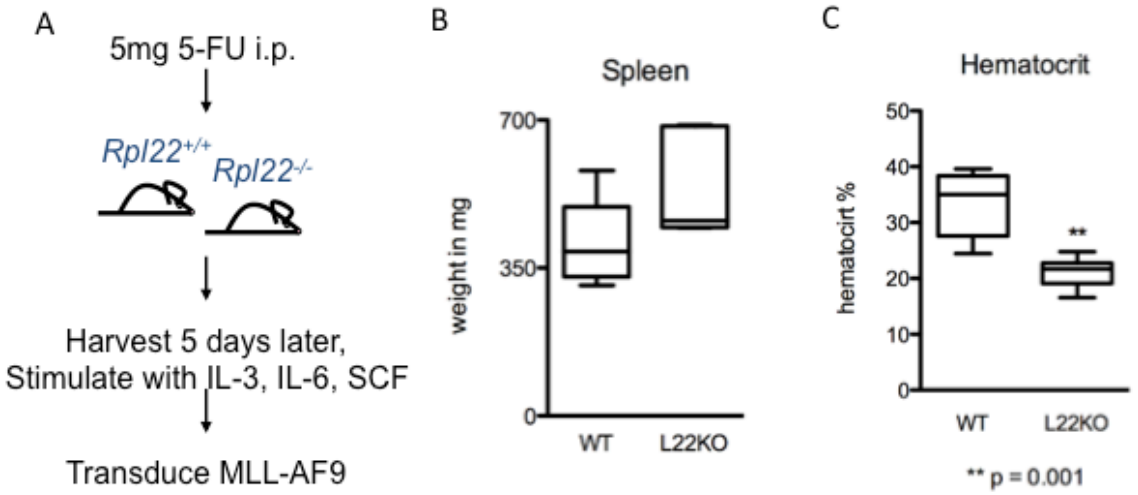

Figure 5 A. Schematic to show the transplant mouse model. BM cells were harvested, transduced with MLL-AF9 oncogene and then transplanted to irradiate recipient mice. B. Spleen weight of the recipient mice receiving transformed BM cells with MLL-AF9. C. Hematocrit of recipient mice receiving BM cells transduced with MLL-AF9.

We used MLL-AF9, a frequent translocation in AML and its oncogenic activity is established in murine model(11, 12). Mice transplanted with MLL-AF9 cells displayed leukemia phenotype as early as 6 weeks(13). So we transduced BM cells from Rpl22-/- and Rpl22+/+ mice with MLL-AF9 and transplanted the cells into the recipient mice. As we expected, mice receiving Rpl22

KO cells display AML phenotype as shown in Fig.5. Rpl22 loss seems to be predisposed to AML.

We also generated MLL-AF9 transgenic mice with Rpl22 KO to study whether Rpl22 deletion will accelerate AML development *in vivo*. We found Rpl22 knockout MLL-AF6 mice displayed poor survival compared with wild type counterpart (Rpl22-/-;MLL-AF9 *VS* Rpl22+/+;MLL-AF9, $p<0.05$)(Fig.6).

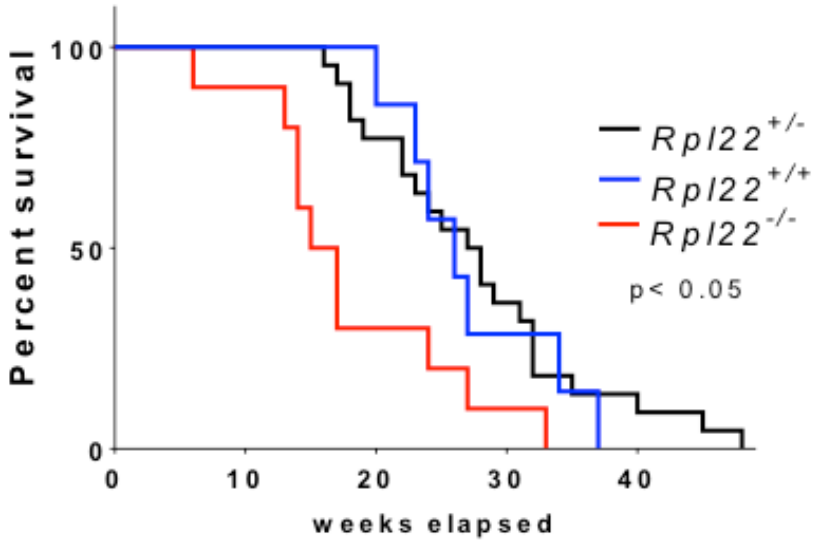

Figure 6. Percentage survival of MLL-AF9 transgenic mice and Rpl22 knockout (Rpl22-/-) mice showed poor survival compared with Rpl22 wild type mice (Rpl22+/+).

KEY RESEARCH ACCOMPLISHMENTS

1. Linked Rpl22 defect with ER stress PERK pathway and thereby increase oncogenic targets
2. Although p53 induction occurs upon ribosomal defects and is usually linked with bone marrow disease, our data doesn't support p53 is the key player in MDS/AML caused by Rpl22 defect.
3. Uncovered a pathological role of Rpl22 defect in promoting AML progression.

CONCLUSION

Provided insight of the mechanism underlying Rpl22 defect in MDS/AML and linked it with ER stress PERK signaling. Our survival data in MLL-AF9 transgenic mice strongly suggest that Rpl22 mutation or deletion might be a poor prognostic marker in AML. On the other hand, because we found Like1 is induced upon Rpl22 inactivation and its expression is required and sufficient to promote transformation and Lin28B induction, we plan to investigate the role of Like1 induction in the development and progression of blood malignancies as well as solid tumors. Also we will evaluate the prognostic value of Rpl22 defect and/or Like1 overexpression in cancer.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

a. Publications

Rao S, Cai KQ, Stadanlick JE, Greenberg N, Solanki-Patel N, Testa JR, Wiest DL. Control lymphoma dissemination by a ribosomal protein Rpl22. *Cancer Research*. 2015 **(submitted)**

Rao S, Zhang Y, Fahl S, Peri S, Rhodes M, Cooper H, Kennedy BK, Rhodes J, Cai KQ, Testa JR, Wiest DL. 2015. Rpl22 inactivation predisposes to transformation through the induction of its proto-oncogene paralog Rpl22-Like1. *Cancer Cell*. 2015. (**to be submitted)**

b. Abstracts Presented in Conference

Rao S, Stadanlick JE, Cai KQ, Wiest DL. 2014 Loss of Rpl22 promotes tumor progression through regulation of angiogenesis and dissemination. AACR- Hematologic Malignancies Conference, Philadelphia, USA (Travel Award)

Greenberg N, Patel NS, Peri S, **Rao S**, Rhodes M, Wiest DL. 2014 Role of Ribosomal Protein Rpl22 in regulating leukemic transformation. AACR-Hematologic Malignancies Conference, Philadelphia, USA

INVENTIONS, PATENTS, AND LICENSES

Nothing to report.

REPORTABLE OUTCOMES

Developed Like1 antibody and tested for the application of western blot, immunoprecipitation, immunofluorescence and immunohistochemistry (IHC) in tissue microarray.

OTHER ACHIEVEMENTS

Nothing to report.

TRAINING OR FELLOWSHIP AWARDS

I joined Dr. David Wiest's lab for postdoctoral training in 2010 to study the mechanism by which inactivation of the ribosomal protein Rpl22 predisposes blood malignancies. Dr. Wiest's expertise in preclinical animal models of T cell malignancy as well as in the manipulation of development of primary hematopoietic stem cells in vitro and in vivo, has enabled me to significantly expand my technical repertoire. In my pursuit of these studies, I have developed a strong interest in hematologic malignancies. Under Dr. Wiest's sponsorship I have gone through the following trainings, which is necessary for an independent position in academia: 1) Writing Skills – I have enrolled in the interactive 6wk grant writing course at Fox Chase and improved my writing skills. Additionally, I am preparing two manuscripts (one submitted to *Cancer Research*). This entails several rounds of review and revision with Dr. Wiest, which also helps to improve my writing ability. 2) Oral Presentations – I have won awards for my oral presentations; I also attended weekly Wiest lab meetings, the postdoctoral seminar series, weekly departmental journal club and Fox Chase Research Festival to further improve my speaking ability 3) Research Networking – our weekly departmental seminar series where I am able to meet with the outstanding speakers that visit Fox Chase Cancer Center and scientific meetings. I attended 2014 AACR-Hematologic Malignancies Conference and 2015 AACR annual conference in Philadelphia. Will also attend ASH meeting in December 2015. All these experiences ensure that I received a well-rounded training experience and will be fully prepared me for independence as a researcher in blood malignancies. In the coming year, Dr. Wiest will continue his rigorous mentorship and support to guide me toward research independence.

REFERENCES

1. Zhang Y, *et al.* (2013) Control of hematopoietic stem cell emergence by antagonistic functions of ribosomal protein paralogs. *Dev Cell* 24(4):411-425.
2. Narla A & Ebert BL (2010) Ribosomopathies: human disorders of ribosome dysfunction. *Blood* 115(16):3196-3205.
3. Rao S, *et al.* (2012) Inactivation of ribosomal protein L22 promotes transformation by induction of the stemness factor, Lin28B. *Blood* 120(18):3764-3773.
4. O'Leary MN, *et al.* (2013) The ribosomal protein Rpl22 controls ribosome composition by directly repressing expression of its own paralog, Rpl22l1. *PLoS Genet* 9(8):e1003708.
5. Hotamisligil GS (2010) Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell* 140(6):900-917.
6. Eckerle S, *et al.* (2009) Gene expression profiling of isolated tumour cells from anaplastic large cell lymphomas: insights into its cellular origin, pathogenesis and relation to Hodgkin lymphoma. *Leukemia* 23(11):2129-2138.
7. Andersson A, *et al.* (2007) Microarray-based classification of a consecutive series of 121 childhood acute leukemias: prediction of leukemic and genetic subtype as well as of minimal residual disease status. *Leukemia* 21(6):1198-1203.
8. Ferreira AM, *et al.* (2014) High frequency of RPL22 mutations in microsatellite-unstable colorectal and endometrial tumors. *Hum Mutat* 35(12):1442-1445.
9. Bai D, Zhang J, Xiao W, & Zheng X (2014) Regulation of the HDM2-p53 pathway by ribosomal protein L6 in response to ribosomal stress. *Nucleic Acids Res* 42(3):1799-1811.
10. Liu Y, *et al.* (2014) Ribosomal protein-Mdm2-p53 pathway coordinates nutrient stress with lipid metabolism by regulating MCD and promoting fatty acid oxidation. *Proc Natl Acad Sci U S A* 111(23):E2414-2422.
11. Chen W, *et al.* (2008) Malignant transformation initiated by Mll-AF9: gene dosage and critical target cells. *Cancer cell* 13(5):432-440.
12. Chen W, *et al.* (2006) A murine Mll-AF4 knock-in model results in lymphoid and myeloid deregulation and hematologic malignancy. *Blood* 108(2):669-677.
13. Xu SM, *et al.* (2013) [Establishment of the retrovirus-mediated murine model with MLL-AF9 leukemia]. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 21(5):1126-1132.

APPENDICES

1. Abstract of manuscript submitted to *Cancer Research*
2. Abstract that will be submitted to *Cancer Cell*

Control of T Lymphoma Dissemination by ribosomal protein, Rpl22

Shuyun Rao[1], Kathy Q. Cai[1], Jason E. Stadanlick[1], Noa Greenberg[1], Nehal Solanki-Patel[1], Sang-Yun Lee[1], Joseph R. Testa[2], and David L. Wiest[1,3]

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Running Title: Role for Rpl22 in T cell lymphoma dissemination

Keywords: Ribosomal proteins, T lymphoma, Rpl22, lymphoma progression, S1P1 receptor

Word Count: 3435

Conflict of Interest: The authors have no potential conflicts of interest to disclose.

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Abstract

Mutations in ribosomal proteins often cause bone marrow failure syndromes associated with increased cancer risk, but in most cases, the basis by which they do so remains unclear. We have previously reported that the ribosomal protein Rpl22 is a tumor suppressor in T cell acute lymphoblastic leukemia/lymphoma (T-ALL), and that loss of just one *Rpl22* allele accelerates T-cell lymphomagenesis by activating NF-κB and inducing the stem cell factor Lin28B. Here, we show that, paradoxically, loss of both alleles of *Rpl22* restricts lymphoma progression through a distinct effect on migration of malignant cells out of the thymus. Lymphoma-prone AKT-transgenic or PTEN-deficient mice on an $Rpl22^{-/-}$ background developed significantly larger and markedly more vascularized thymic tumors than those observed in $Rpl22^{+/+}$ control mice. But, unlike $Rpl22^{+/+}$ or $Rpl22^{+/-}$ tumors, $Rpl22^{-/-}$ lymphomas were retained in the thymus as large mediastinal masses that did not disseminate to the periphery, but were eventually lethal, presumably due to suffocation caused by impingement of the lungs by the lymphoma. We traced the defect in *the* $Rpl22^{-/-}$ lymphoma migratory capacity to downregulation of the KLF2 transcription factor and consequent reduction in expression of the KLF2 target and key migratory factor sphingosine 1-phosphate receptor 1 (S1P1 receptor). Indeed, re-expression of the S1P1 receptor in Rpl22-deficient tumor cells restores their migratory capacity. Collectively, these data reveal that, while loss of one copy of *Rpl22* promotes lymphomagenesis and disseminated disease, loss of both copies limits malignant cells to the thymus via a novel S1P1-receptor dependent effect on migration.

Rpl22 inactivation predisposes to transformation through induction of its proto-oncogenic paralog Rpl22-Like1

Shuyun Rao[1], Yong Zhang[1], Shawn Fahl[1], Suraj Peri[1], Michele Rhodes[1], Noa Greenberg[1], Harry S. Cooper[3], Brian K. Kennedy[4], Jenifer Rhodes[1], Kathy Q. Cai[2], Joseph Testa[2], and David L. Wiest[1,5]

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Summary (120 words)

While ribosomal protein (RP) paralogs are pretty common in lower organisms, only few are conserved in vertebrates with unclear functions independent of ribosomal biogenesis. Among these few is *Rpl22l1* (*Like1*), a paralog of *Rpl22* whose expression is markedly induced upon the inactivation of *Rpl22*, which is associated with increased transformation potential. Here we revealed that LIKE1, normally repressed by RPL22 in a post-transcriptional manner but induced by *RPL22* loss, is both necessary and sufficient to promote transformation. Moreover, LIKE1 induction is observed in a number of human cancers and is positively associated with poor survival in colon cancer. In summary, our data suggest LIKE1 induction facilitate oncogenesis and might be a valuable prognostic indicator for poor survival in colon cancer.